PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Linda M. Pacioretty *et al.*
Application No.:	10/699,195
Filing Date:	10/31/2003
Docket Number:	CLANACCR_001NP
Title:	**COMPOSITIONS AND METHODS FOR THE TREATMENT OF HIV-ASSOCIATED FAT MALDISTRIBUTION AND HYPERLIPIDEMIA**
Examiner:	Chong, Young Soo
Art Unit:	1617

CERTIFICATE OF TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service as "EXPRESS MAIL" MAILING LABEL NUMBER **EO 972 499 599 US** in an envelope addressed to MAIL STOP RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date indicated below.

Date: 3/8/10

John G. Babish

MAIL STOP RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Sir:

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

I, John G. Babish declare as follows:

1) I am Dr. John G. Babish, Chairman, Bionexus, Ltd. I have held this position since June 1997.

2) I have Doctorate and Masters degrees, respectively, in Biochemistry and Chemistry from Cornell University, as well as a Bachelor degree in Biochemistry from The Pennsylvania State University. A copy of my Curriculum Vitae is attached as Exhibit A.

3) I am also an inventor named in 50 domestic patent applications including U.S. Application Nos. 10/699,195; 10/141,085; 10/789,814; 10/789,817; 10/988,393; 10/480,145; 10/484,123; 10/881,404; 10/774,048; 10/464,834; 10/234,002 and 09/952,632 and 16 issued domestic patents, including U.S. Patent Nos. 7, 279,185; 7,270,835; 6,733,793; 6,140,063; 5,506,420; 6,629,835; 6,733,793 and 6,908,630.

4) On the basis of 30 years of training and experience, I am an expert in the art of molecular biology of pharmaceuticals and xenobiotics. Prior to my retirement, I was a faculty member at the College of Veterinary Medicine, Cornell University for 17 years. As Professor of Pharmacology and Toxicology, my research program involved the elucidation of mechanisms by which xenobiotics affect signaling pathways in normal and transformed cells. Using the tools of molecular biology such as monoclonal antibodies, northern and western blotting and enzyme-linked immunoassays, my research program developed cell-based assays for the identification of small molecules directed at inhibiting selected cellular functions. Findings from these studies were used to identify potential anti-viral and anti-neoplastic pharmacophores from natural products. My research has also identified both positive and negative drug-drug and drug-nutrient interactions.

5) Since 1999, I have performed various anti-oxidant screening studies using both cell-free and whole cell models. Over the years my laboratory has screened over 700 compounds; the results of several of these studies utilizing whole cell models and performed prior to the filing of the above referenced application are summarized herein as Exhibit B for the oxidant-stressed Jurkat and RAW 264.7 cell models and Exhibit C for the oxidant-stressed HAEC cell model. Methods used in these studies are detailed in Exhibit D.

7) I understand that in the course of the teleconference conducted on January 14, 2010, Examiner Young Soo Chong requested a Declaration of the side-by-side ant-

oxidant studies conducted by the Applicants to demonstrate the lack of functional equivalence between NAC and CoQ10.

8) *Conclusion.* Based upon whole cell studies in oxidant-stressed Jurkat, RAW 264.7 and HAEC cells, NAC and CoQ10 are not functionally equivalent anti-oxidants and this was know to the Applicants prior to the filing of the referenced application (Tables 1 and 2 in Exhibits B and C, respectively).

Note also that the HACE cells used by the Applicants, where NAC demonstrated highly active anti-oxidant activity (9.9 μg/mL) and CoQ10 was inactive (Table 2, Exhibit C), was the same cell line used in Medford. Thus, it would not be obvious for the Applicants to substitute CoQ10 for NAC as functionally equivalent anti-oxidants, as the Applicants knew these two compounds were not functionally equivalent prior to the instant application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 3/8/10

John G. Babish, Ph.D.
Chairman, Bionexus, Ltd.
Cornell Technology Park
30 Brown Road
Ithaca, NY 14850

Exhibit A

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

John G. Babish — Chairperson, BIOnexus, Ltd.
Executive Vice President, MetaProteomics Inc.

Education

Institution and Location of Study	Degree	Date Conferred	Field
The Pennsylvania State University, State College, PA	B.S.	1968	Biochemistry
Cornell University, Ithaca, NY	M.S.	1974	Chemistry
Cornell University, Ithaca, NY	Ph.D.	1976	Biochemistry

Research and Professional Experience

Aug. 2002 – present — Executive Vice President of Research & Development, MetaProteomics, Research Laboratories, Ithaca, NY. Metaproteomics develops clinically proven, patented dietary supplements and pharmaceuticals from natural sources. Duties include the design and evaluation of experiments elucidating mechanism of action and biological activity within complex mixtures.

1998 – present — (5% Effort) National Coordinator for the USDA Minor Species Drug Program (NRSP-7). The NRSP-7 program is funded by the USDA to provide funds and expertise necessary for the approval of pharmaceuticals used in the treatment of diseases associated with minor crop species. Duties include the coordination of industrial, academic and regulatory resources necessary for protocol development through final drug approval.

1997 – present — Co-founder and Chairperson of BIOnexus, Ltd. Ithaca, NY. BIOnexus develops and markets nutritional supplements to address health problems associated with AIDS. NutriVir™, the BIOnexus supplement for wasting in HIV/AIDS, generated approximately $600,000 in gross revenues in its first year of sales. NutriVir™ is reimbursed by Medicaid in 14 states.

1991 – 1996 — Founder, Chairperson, President and CEO of Paracelsian, Inc., Ithaca, NY. The Company was launched from the technology transfer program of Cornell University in 1991, and with the public offering in 1992 (Nasdaq, PRLN), became the first public corporation of a Cornell University technology transfer effort. Babish was associated

	with the attainment of over $12 million dollars in corporate financing.
1984 – 1996	Tenured, Associate and Professor of Pharmacology and Toxicology, Department of Pharmacology, College of Veterinary Medicine, Cornell University. Offered the first course in molecular risk assessment in the USA in 1979; member of the graduate Fields of Pharmacology, Toxicology, Veterinary Medicine, Food Science and Epidemiology; successfully petitioned the State of New York for the approval of the separate Fields of Toxicology and Pharmacology at Cornell University.
1978 – 1984	Assistant Professor, Department of Preventive Medicine, NYS College of Veterinary Medicine, Cornell University, Ithaca, NY.
1976 – 1978	Postdoctoral Scientist, Food and Drug Research Labs, Waverly, NY.

Invited Presentations (Recent of 38 presentations)

Micronutrient deficiencies in AIDS wasting at Progressive Management of AIDS Wasting: 2000. Hunter College, NYC. March 24, 2000.

Phytochemicals and NF-kB activation at IBC's Conference on The Health Benefits of Natural Phytoceuticals. Montreal Bonaventure Hilton, July 22 – 23, 1997.

Chemically-induced cell cycle stasis in immunotoxicology. 12th Annual NIOSH Conference on Mechanisms of Immunotoxicology – Role of Apoptosis in Immunotoxicology. University of West Virginia, Morgantown, WV. September 10 – 12, 1997.

Publications (Selected of 108 peer-reviewed publications)

Hall AJ, Tripp M, Howell T, Darland G, Bland JS, Babish JG. (2006) Gastric mucosal cell model for estimating relative gastrointestinal toxicity of non-steroidal anti-inflammatory drugs. *Prostaglandins Leukot Essent Fatty Acids.* 75(1):9-17.

Payne M.A., Babish J.G., Bulgin M., Lane M., Wetzlich S., Craigmill A.L. (2002) Serum pharmacokinetics and tissue and milk residues of oxytetracycline in goats following a single intramuscular injection of a long-acting preparation and milk residues following a single subcutaneous injection. J Vet Pharmacol Ther. 25(1):25-32.

Calabrese C., Berman S.H., Babish J.G., et al. (2000) A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytother Res. 14(5):333-338.

Ma,X., Stoffregen,D.A., Wheelock,G.D., Rininger,J.A. and Babish,J.G. (1997) Discordant hepatic expression of the cell division control enzyme p34cdc2 kinase, proliferating cell nuclear antigen, p53 tumor suppressor protein, and p21Waf1 cyclin-dependent kinase inhibitory protein after WY14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid) dosing to rats. Mol. Pharmacol., 51, 69-78.

Rininger,J.A., Goldsworthy,T.L. and Babish,J.G. (1997) Time course comparison of cell-cycle protein expression following partial hepatectomy and WY14,643-induced hepatic cell proliferation in F344 rats. Carcinogenesis, 18, 935-941.

Rininger,J.A., Stoffregen,D.A. and Babish,J.G. (1997) Murine hepatic p53, RB, and CDK inhibitory protein expression following acute 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure. Chemosphere, 34, 1557-1568.

Rininger,J.A., Wheelock,G.D., Ma,X. and Babish,J.G. (1996) Discordant expression of the cyclin-dependent kinases and cyclins in rat liver following acute administration of the hepatocarcinogen [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (WY14,643). Biochem. Pharmacol., 52, 1749-1755.

Vancutsem,P.M. and Babish,J.G. (1996) In vitro and in vivo study of the effects of enrofloxacin on hepatic cytochrome P-450. Potential for drug interactions. Vet. Hum. Toxicol., 38, 254-259.

Patents (Selected of 16 US and three foreign patents)

US Patent No. **7,279,185**	Curcuminoid compositions exhibiting synergistic inhibition of the expression and/or activity of cyclooxygenase-2.
US Patent No. **6,733,793**	Oral composition with insulin-like activities and methods of use.
US Patent No. **5,833,994**	Use of the Ah receptor and Ah receptor ligands to treat or prevent cytopathicity of viral infection.
US Patent No. **5,612,188**	Automated, multicompartmental cell culture system.
US Patent No. **5,529,899**	Immunoassay for Ah receptor transformed by dioxin-like compounds.
US Patent No. **5,496,703**	Indirect immunoassay for dioxin-like compounds

Exhibit B

Table 1. Anti-oxidant Activity of 60 Compounds Tested in Oxidant-stressed Jurkat and RAW 264.7 Cell Models

Compound	Date	Jurkat H_2O_2 + LPS IC_{50} ± 95% CI [μg/mL]	Date	RAW H_2O_2 + LPS IC_{50} ± 95% CI [μg/mL]
Acetaminophen -Sigma	5/9/02	No activity ≤ 30 μg/mL	5/15/02	8.6 (5.0 - 15)
Alpinia galanga rhizomes (10:1 extract)	5/17/02	No activity ≤ 30 μg/mL	5/17/02	17 (14 - 22
Arginine - Sigma	4/29/02	No activity ≤ 25 μg/mL	4/30/02	No activity ≤ 30 μg/mL
Ascorbic acid - Metagenics (RM07453)	4/11/02	5.7 (3.6 - 8.9)	4/24/02	~2.4
Aspirin - Sigma	4/30/02	No activity ≤ 30 μg/mL	4/30/02	No activity ≤ 30 μg/mL
Astaxanthin - Sigma	4/30/02	No activity ≤ 30 μg/mL	4/30/02	48 (20 - 112)
AZT (Zidovudine) - Sigma	4/8/02	No activity ≤ 30	5/2/02	No activity ≤ 30 μg/mL
Berberine - Sigma	5/1/02	No activity ≤ 30 μg/mL	5/1/02	25 (7.3 - 87)
Berberine 30% - Harten Corporation	4/29/02	Pro-oxidant @ 25 μg/mL	4/23/02	No activity ≤ 30 μg/mL
Bergenin	5/1/02	No activity ≤ 30 μg/mL	5/1/02	No activity ≤ 30 μg/mL
Boswellin (RM07781)	4/5/02	Pro-oxidant @ 25 μg/mL	4/24/02	No activity ≤ 25 μg/mL
BoVin Grape Extract - Cyvex Nutrition	4/8/02	0.21 (0.12 - 0.35)	4/25/02	4.1 (2.8 - 6.0)
Caffeic acid (COX2 inhibitor)	4/29/02	3.2 (2.5 - 4.1)	4/26/02	3.3 (2.1 - 5.4)
Carnosic acid (Rosemary)	4/30/02	17 (13 - 22)	4/30/02	8.8 (6.6 - 12)
CAROMIX PLUS™ - H. Reisman	4/1/02	12 (10 - 13)	4/25/02	No activity ≤ 25 μg/mL
Carvacrol - Aldrich	5/10/02	No activity ≤ 30 μg/mL	5/10/02	No activity ≤ 30 μg/mL
Catechin - Sigma	4/2/02	~0.13	4/25/02	4.9 (3.6 - 6.7)
Cayenne Pepper - Metagenics (RM06572)	4/9/02	No activity ≤ 25 μg/mL	4/24/02	No activity ≤ 25 μg/mL
Cell Protect - Metagenics (C20310132)	4/8/02	1.5 (1.2 - 1.9)	5/2/02	15 (7.8 - 30)
Chlorogenic acid	4/12/02	1.0 (0.75 - 1.5)	4/25/02	4.1 (3.8 - 4.5)
Citrulline - Metagenics (077692)	4/9/02	Pro-oxidant ≥ 12.5 μg/mL	4/23/02	No activity ≤ 30 μg/mL
CoQ10 - Sigma	**5/1/02**	**No activity ≤ 30 μg/mL**	**5/1/02**	**Pro-oxidant @ 30 μg/mL**
Curcumin - Metagenics (07367)	4/8/02	0.24 (0.13 - 0.45)	4/19/02	4.5 (2.0 - 10)
Curcumin granular - Metagenics (06656)	4/11/02	2.4 (1.6 - 3.6)	4/24/02	5.0 (4.9 - 5.1)
Ellagic acid	4/29/02	≤3.13	4/26/02	2.9 (1.3 - 6.4)
Emodin	4/11/02	26 (25 - 28)	5/2/02	No activity ≤ 30 μg/mL
Emu oil (Emu Gold)	5/8/02	No activity ≤ 30 μg/mL	5/8/02	No activity ≤ 30 μg/mL
Ergothionenine - Sigma	5/1/02	~29	5/1/02	No activity ≤ 30 μg/mL
Fisetin	4/29/02	1.5 (0.44 - 5.1)	4/26/02	4.2 (2.2 - 7.8)
Galangin - Sigma	4/2/02	~13	4/25/02	4.0 (3.4 - 4.9)
Garlic - A1500 Powder Basic Vegetable Products	4/30/02	No activity ≤ 30 μg/mL	4/30/02	No activity ≤ 30 μg/mL
Ginger - Metagenics (07782)	4/11/02	No activity ≤ 30	4/24/02	No activity ≤ 25 μg/mL
Ginger Root - Metagenics (06936)	4/8/02	18 (15 - 22)	4/19/02	No activity ≤ 25 μg/mL
Glutamine - Aldrich	5/10/02	No activity ≤ 30 μg/mL	5/10/02	Pro-oxidant ≥ 15 μg/mL
Glutathione - Sigma	5/1/02	No activity ≤ 30 μg/mL	5/1/02	No activity ≤ 30 μg/mL
Green Tea Extract - Metagenics (RM06630)	4/9/02	0.13 (0.07 - 0.25)	4/25/02	3.1 (2.9 - 3.5)
Histidine - Sigma	4/30/02	Pro-oxidant ≥ 7.5 μg/mL	4/30/02	No activity ≤ 30 μg/mL
Ibuprofen - Sigma	4/30/02	No activity ≤ 30 μg/mL	4/30/02	31 (15 - 65)
Inflavonoid IC tablets - Metagenics	4/9/02	2.1 (2.0 - 2.2)	4/24/02	17 (7.6 - 36)
Isoleucine - Sigma	5/10/02	No activity ≤ 30 μg/mL	5/10/02	No activity ≤ 30 μg/mL
Keampferol - Sigma	4/2/02	11 (11 - 12)	4/25/02	9.3 (8.1 - 10)
Limonene - Metagenics	4/9/02	No activity ≤ 25 μg/mL	4/23/02	No activity ≤ 25 μg/mL
Lipoic acid - Sigma	4/2/02	Pro-oxidant ≥ 3.13 μg/mL	4/24/02	Pro-oxidant ≤3.13 μg/mL
Lycopene	4/11/02	No activity ≤ 30	5/9/02	No activity ≤ 30
Methionine - Sigma	5/10/02	No activity ≤ 30 μg/mL	5/10/02	No activity ≤ 30 μg/mL
Mitochondrial Resuscitate	4/29/02	37	4/26/02	No activity ≤ 30 μg/mL
Morin - Sigma	4/29/02	1.0 (0.54 - 2.3)	4/26/02	~2.8
Myricetin	5/1/02	7.9 (4.0 - 15)	5/1/02	6.0 (3.3 - 11)
N-Acetylcysteine - Sigma	**4/1/02**	**Pro-oxidant ≥ 3 μg/mL**	**4/24/02**	**19.0 (18.7 - 19.6)**
Naringenin	4/29/02	No activity ≤ 25 μg/mL	4/26/02	No activity ≤ 25 μg/mL
Quercetin - Metagenics (07346)	4/8/02	0.39 (0.23 - 0.67)	4/23/02	2.7 (2.2 - 3.2)
Quercetin - Metagenics (07671)	4/11/02	3.2 (1.9 - 5.3)	4/24/02	3.1 (1.8 - 5.3)
Resorcinol	4/29/02	No activity ≤ 25	4/26/02	No activity ≤ 25
Resveratrol - Sigma	4/2/02	Pro-oxidant ≥ 6.25 μg/mL	4/23/02	~3.0
Rosemary - Metagenics (07720)	4/8/02	1.5 (1.0 - 2.3)	4/23/02	8.3 (1.9 - 37)
Rutin - Metagenics (06394)	4/8/02	1.29 (0.58 - 2.86)	4/23/02	3.5 (2.8 - 4.4)
Se-(Methyl)-Selenocysteine Hydrochloride - Sigma	5/10/02	Pro-oxidant @ 7.5 μg/mL	5/10/02	Pro-oxidant ≥ 3.75
Selenate, sodium - Sigma	5/10/02	No activity ≤ 30 μg/mL	5/10/02	Pro-oxidant @ 30 μg/mL
Seleno-L-methionine - Sigma	5/10/02	Pro-oxidant @ 7.5 μg/mL	5/10/02	No activity ≤ 30 μg/mL
ß-Carotene - Sigma	4/1/02	26 (13 - 53)	5/2/02	No activity ≤ 30 μg/mL

Exhibit C

Table 2. Anti-oxidant Activity of 25 Compounds Tested in the Oxidant-stressed HAEC Model

Compound	Date	HAEC/1000 μM H_2O_2 IC_{50} [μg/mL]	HAEC/1000 μM H_2O_2 95% CL [μg/mL]
Trolox (Vitamin E activity)	5/28/03	0.58	(0.20 - 1.7)
BoVin Grape Extract - Cyvex Nutrition	5/28/03	1.9	(0.75 - 4.7)
Curcumin - Metagenics (07367)	5/28/03	8.5	(6 - 12)
N-acetylcysteine-Sigma	5/28/03	9.9	(4.8 - 13)
Hops Powder Extract	5/21/03	24	(18 - 32)
Cell Protect - Metagenics (C20310132)	5/28/03	24	(1- - 56)
Ascorbic acid - Metagenics (RM07453)	5/28/03	26	(11- 64)
Hopsteiner Reduced Iso Extract 35% (AN1078)	5/21/03	35	(7- 169)
Hopunion Whole Hop Extract (AN1040)	5/21/03	37	(25 - 55)
Hops Xtr Powder (AN1077)	5/21/03	39	(7 - 213)
Hopsteiner Ethanol Extract (AN1081)	5/21/03	39	(15 - 99)
Hops Liquid CO2 Extract	5/21/03	41	(31 - 53)
Hops Dry Powder (Microcap)	5/21/03	70	(24 - 203)
Hopsteiner CO2 Hop Extract	6/2/03	71	(15 - 340)
YC-AlphaRich	6/2/03	76	(59 - 97)
Hops Microcap (AN1073)	5/21/03	96	(10 - 905)
α-Tocopherol succinate - Sigma	5/28/03	>100	Not Computed
Astaxanthin - Sigma	5/28/03	>100	Not Computed
CAROMIX PLUS™ - H. Reisman	5/28/03	>100	Not Computed
CoQ10 - Sigma	5/28/03	>100	Not Computed
Hopsteiner Beta Aroma Extract Light Stable	6/2/03	>100	Not Computed
Mg Rho 60 (RIAA)	6/2/03	>100	Not Computed
RIAA Hops #1199	6/2/03	>100	Not Computed
Watertown 5% alpha-hydrohop	6/2/03	>100	Not Computed
Watertown 5% beta-hydrohop	6/2/03	>100	Not Computed

>100 indicates no activity in this model.

Exhibit D

SCREENING ANTIOXIDANT ACTIVITY OF SELECT COMPOUNDS IN HUMAN T CELLS, MURINE MACROPHAGES, AND HUMAN AORTIC ENDOTHELIAL CELLS

TEST MATERIALS AND CHEMICALS:
Commercial dietary supplements were obtained from Metagenics (Gig Harbor, WA) or Sigma (St. Louis, MO). Heat inactivated Fetal Bovine Serum (FBS-HI Cat. #35-011CV) and Dulbecco's Modification of Eagle's Medium (DMEM Cat #10-1013CV) was purchased from Mediatech (Herndon, VA). Unless otherwise noted, all standard reagents were obtained from Sigma (St. Louis, MO) and were the purest commercially available.

OBJECTIVE
It was the objective of these studies to assess the antioxidant activity of selected nutrients, dietary supplements in oxidant-stressed cell lines.

METHODS
Cell culture and treatment with test material – Jurkat cells (human T cells) and RAW 264.7 cells (murine macrophages) were obtained from the American Type Culture Collection (Manassas, VA) and human aortic endothelial cells (HAEC) were obtained from Cambrex (Walkersville, MD). All cell lines were subcultured according to supplier instructions.

For experiments, cells were grown in a T75 flask at 37°C in a humidified 95% air/5% CO_2 atmosphere. Prior to cells becoming confluent; they were prepared for testing in microtiter plates by treating with a fresh trypsin solution and counted. Approximately 10^5 cells/well are aliquoted into 96-well plates in 200 µL growth medium per well. Cells were allowed to reach 80% confluence before treatment with test material and H_2O_2 stressor solutions.

On the day of the experiment, the growth medium was aspirated and replaced with 200 µL media containing the test material. The growth medium containing test material was formulated by adding 4 µL of 250X stock test material in DMSO to 1 mL of growth media. Thus, each well contained the same amount of DMSO. Control wells received DMSO in growth media only. The final concentrations of test material for samples tested on were 100, 10, 1, and 0.1 µg/mL.

Anti-oxidant assay
Oxidative stress was measured in using dichlorofluorescein diacetate (DCFH-DA) in a microtiter plate assay as previously described [Wang, H. and Joseph, J.A. (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology & Medicine 27:612-616]. Based upon the conversion of the non-fluorescent 2',7'-dichlorofluorescin ($DCFH_2$) to the highly fluorescent 2',7'-dichlorofllurorescein (DCF) by various free radicals, this indiscriminate probe produces concentration dependent changes in cellular fluorescence. Thus, the assay can be used to quantify overall oxidative stress in cells.

The extracts were added to the wells at four concentrations with two replicates per concentration. DMSO was added to the control wells in an equal volume to that contained

with the test materials. Fifteen minutes after the addition of the test compounds, H_2O_2 was added to each well in a volume of 20 μL to achieve a final concentration of 1000 μM.

All fluorescent (RFU) measurements were preformed in a 96-well polypropylene plate with stirring; temperature was maintained at 37°C. The excitation filter was set at 485 nm and the emission filter was set at 530 nm. Fluorescence for each well was captured, digitized and stored on a computer using Cytofluor (Version 4.0). Data points were taken every 10 minutes for 60 minutes. Complete assays were exported to an Excel (Microsoft, Seattle, WA) spreadsheet for analysis.

The extent of oxidation of intracellular $DCFH_2$ during the 60-minute duration of the experiment was expressed as $AUC_{(0-60)}$ and calculated using the trapezoidal method. The area for each 10-minute reading period generated by the increase in RFU over time is trapezoidal. Summation of the area of each trapezoid ($A_{(a-b)} = [0.5*(RFU_b+RFU_a)]*[T_b-T_a]$, where T_b-T_a represents the ten minute interval between RFU readings from 0 to 60 minutes) was used to compute the $AUC_{(0-60)}$.

Inhibition of $DCFH_2$ oxidation by test materials was calculated based upon $AUC_{(Cells+DCFH2+H2O2+LPS)}$ = 0% inhibition and $AUC_{(Cells+DCFH2)}$ = 100% inhibition and $AUC_{(Cells)}$ = background. The dynamic range of inhibition was defined as the AUC between 0 and 100% = $AUC_{(Cells+DCFH2)} - AUC_{(Cells+DCFH2+H2O2+LPS)}$. The fractional inhibition of $DCFH_2$ oxidation by each concentration of test material was calculated as:

$$\frac{[AUC_{(Cells+DCFH2+H2O2+LPS+Test\ Sample\ Dose)} - AUC_{(Cells+DCFH2+H2O2+LPS)}]}{[AUC_{(Cells+DCFH2)} - AUC_{(Cells+DCFH2+H2O2+LPS)}]}$$

Cell viability – Cell viability was assessed by microscopic inspection of cells prior to or immediately following recording of fluorescence. Cell mortality was noted when observed.

STATISTICAL METHODS

Calculations – A minimum of four concentrations (Tables 1A, 1B, 1C and 1D) over three independent assays was used to compute dose-response curves and medium inhibitory concentrations (IC_{50s}) with 95% confidence intervals using CalcuSyn (BIOSOFT, Ferguson, MO). This statistical package performs multiple drug dose-effect calculations using the Median Effect methods described by T-C Chou and P. Talaly.

ABBREVIATIONS

CL: confidence limit; DMEM: Dulbecco's Modification of Eagle's Medium; DMSO: dimethylsulfoxide; FBS: fetal bovine serum; FBS-HI: fetal bovine serum, heat inactivated; IC_{50}: median inhibitory concentration.

LITERATURE CITED

Wang, H. and Joseph, J.A. (1999) Quantifiying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology & Medicine 27:612-616.